INFECTION AND IMMUNITY, July 1981, p. 186-192
0019-9567/81/070186-07$02.00/0

Vol. 33, No. 1

Development of a Radioimmunoassay for *Escherichia coli* Heat-Stable Enterotoxin: Comparison with the Suckling Mouse Bioassay

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Received 19 December 1980/Accepted 2 April 1981

Escherichia coli strains which produce heat-stable enterotoxin (ST) are usually identified by demonstrating the production of ST. At present, ST can be detected only be bioassay methods. Recently, we purified *E. coli* ST, which enabled us to develop a radioimmunoassay for ST. Radioiodination of ST was performed by the lactoperoxidase method, which resulted in a high specific activity and retained the biological activity of ST. Anti-ST antisera were raised in goats by injecting the goats with pure ST coupled to bovine immunoglobin G. Antibody titers ranged from 1:8,000 to 1:40,000. Using these reagents, we examined assay conditions thoroughly and found that a 14- to 18-h incubation at 4°C in sodium acetate buffer with an ionic strength of 120 mM (pH 6.2) gave maximal sensitivity and reproducibility. Free ST was separated from antibody-bound ST by dextran-coated charcoal. This radioimmunoassay accurately and reproducibly measured ST in the range from 50 to 500 pg of ST per tube and could quantitate ST accurately in complex bacteriological media. This assay was specific for ST_a, measured human and porcine ST_a equally well, and did not cross-react with ST_b, with several other enterotoxins, or with various gastrointestinal peptides. Intact disulfide bridges in the ST molecule were required for immunoreactive activity.

Enterotoxigenic *Escherichia coli* strains are important causes of diarrhea in humans and in various domestic animals (2, 9, 14, 23). These organisms cause diarrhea by producing either a high-molecular-weight heat-labile enterotoxin or a low-molecular-weight heat-stable (ST) entertoxin (2, 9, 23). *E. coli* heat-labile enterotoxin is immunochemically similar to cholera enterotoxin; both of these compounds cause diarrhea by activating the adenylate cyclase-cyclic adenosine monphosphate system (2, 19). In contrast, *E. coli* may produce one or more ST molecules (3, 10, 18) which reportedly are nonimmunogenic (1, 3, 5, 9, 23). The mechanism by which ST causes diarrhea is uncertain, but it may involve activation of the guanylate cyclase-cyclic guanosine monophosphate system (6, 8, 11, 20).

The epidemiological importance of ST-producing *E. coli* strains, the magnitude of the occurrence of these strains in food (24), and the uncertainty concerning the mechanism of action of ST stem in part from the lack of a simple, accurate, highly sensitive test for ST. At present, *E. coli* strains which produce ST can be identified only by demonstrating that they produce ST. Currently, the only available assay systems for ST are bioassays, and the most commonly used bioassay is the suckling mouse assay (7). However, this method is cumbersome and, at best, only semiquantitative.

Recently, we purified and chemically characterized *E. coli* ST (26). The availability of this pure material enabled us to develop a radioimmunoassay (RIA) for ST, as described below.

(A preliminary presentation of these results has been published previously [R. A. Giannella and K. W. Drake, Gastroenterology **78**:1172, 1980].)

MATERIALS AND METHODS

Preparation and purification of *E. coli* ST. The ST produced by a human strain of *E. coli* (O42:K86:H37) was purified as previously described (26). Briefly, ST was purified from culture media by sequential chromatography through Amberlite XAD, Sephadex G-25, diethylaminoethyl-Sephacel, and Sephadex G-25 columns. Purity was documented by various chemical criteria, including thin-layer chromatography, thin-layer electrophoresis, polyacrylamide gel electrophoresis, amino acid analysis, and amino-terminal analysis. This ST is a peptide containing 18 amino acids, including two tyrosine residues; it is a small, compact, folded molecule and contains at least one

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disulfide bridge which is required for biological activity (26).

The biological activity of ST was quantitated by the suckling mouse assay (7); 1 mouse unit of ST activity was defined arbitrarily as the amount of toxin producing a ratio of intestinal weight to carcass weight of 0.083 (26).

This ST is methanol soluble and active in the suckling mouse assay and neonatal piglets and thus is STa according to the terminology of Burgess et al. (3).

Radioiodination of ST. Pure ST was iodinated with $Na^{125}I$ (specific activity, >350 Ci/ml; Amersham/Searle, Arlington Heights, Ill.) by using a modification of the lactoperoxidase method of Marchalonis (15). In this modification we used immobilized preparations of lactoperoxidase and glucose oxidase in the form of Enzymobeads (Bio-Rad Laboratories, Richmond, Calif.). The iodination procedure was performed in borosilicate glass test tubes (10 by 75 mm). All reagents were dissolved in 0.2 M sodium phosphate buffer (pH 7.2). Reagents were added in the following order: phosphate buffer, 25 μl; 10 μg of pure ST, 25 μl; Enzymobead reagent, 50 μl; 1.0 mCi of $Na^{125}I$, 25 μl; and 1% beta-D-glucose, 25 μl. The total volume in each tube was 150 μl. After 25 min at room temperature, the radioiodination reaction mixture was loaded onto a diethylaminoethyl-Sephacel column (0.9 by 25 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 10 mM bis-tris(hydroxymethyl)aminomethane buffer (pH 6.5). The column was then eluted with a 600-ml linear NaCl gradient (0 to 200 mM) in bis-tris(hydroxymethyl)aminomethane buffer.

Preparation of anti-ST antisera. Antibodies to ST were produced in two goats by immunizing the goats with ST conjugated to bovine immunoglobulin G (IgG). Pure ST was coupled to bovine IgG by the carbodiimide method, as described by McGuigan (16). Briefly, a 10-fold molar excess of pure ST (10 mg in 1.0 ml of 0.05 M potassium phosphate buffer, pH 7.4) was added to bovine IgG (78 mg in 1.0 ml of phosphate buffer); then 3 ml of 0.15 M NaCl containing 125 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added, and the mixture was stirred slowly for 20 h at room temperature. The reaction mixture was dialyzed copiously against 1,000 ml of 0.15 M NaCl-0.01 M potassium phosphate buffer (pH 7.4). The bovine IgG-ST complex was brought to a final volume of 10 ml by adding potassium phosphate buffer (7.8 mg of IgG per ml) and was stored frozen at −20°C until it was used. The same batch of ST-IgG complex was used for all injections and was defrosted and refrozen for each use.

Successful coupling of ST to bovine IgG was documented and the ratio of this coupling was quantitated by adding a tracer quantity of ^{125}I-labeled ST to the reaction mixture. After dialysis, the amount of ^{125}I-labeled ST remaining in the dialysis bag and coupled to IgG was measured, and the number of ST molecules bound to each IgG molecule was calculated. The coupling ratio of ST molecules to IgG molecules was 5.6:1.

Animals were inoculated with the IgG-ST complex in the following manner. Initially, each goat was injected with a 1:1 (vol/vol) suspension of IgG-ST complex in complete Freund adjuvant. Each goat was injected with 0.5-ml portions of the suspension at four separate subcutaneous sites; i.e., each goat received a total of 10.4 mg of IgG. After a 6-week rest period, each goat was boosted monthly with one-half the original quantity of IgG-ST complex without adjuvant injected into the original injection sites. The goats were bled 8 or 9 days after each monthly injection. Each serum was separated by centrifugation, dialyzed against 10 mM sodium acetate buffer (pH 6.2), diluted in this buffer, divided into aliquots, and lyophilized. Antisera were stored at −20°C.

Immunoassay procedure. The immunoassay was performed in 0.12 M sodium acetate buffer (pH 6.2) nonsiliconized borosilicate test tubes (12 by 75 mm). Each tube contained (in order of addition) sodium acetate buffer, 100 μg of bovine serum albumin, 40 to 60 μl of an ST standard or an unknown sample, 100 μl of ^{125}I-labeled ST (8,000 to 15,000 cpm), and 100 μl of antibody in buffer at a dilution (1:20,000 or 1:40,000) sufficient to bind 40 to 60% of the labeled ST. The volume of the complete reaction mixture was 0.5 ml.

Each reaction mixture was incubated at 4°C for 18 to 20 h, and free ST was separated from antibody-bound ST by using dextran-coated charcoal as follows. At the conclusion of the incubation period, 100 μl of a freshly prepared dextran-coated charcoal suspension (0.1% dextran T70 and 1% activated charcoal in 120 mM sodium acetate buffer, pH 6.2) was added to the reaction mixture, and the reaction tube was centrifuged at 3,000 rpm at 4°C for 10 min. The supernatant was transferred to another tube, and both the charcoal pellet and the supernatant were counted with a Packard gamma scintillation spectrometer.

Cross-reactivity studies. To test the specificity of the RIA system, we tested various bacterial enterotoxins for the ability to cross-react with anti-ST antiserum. The purified enterotoxins tested included (i) pure *E. coli* heat-labile enterotoxin (kindly supplied by R. Finklestein, University of Missouri College of Medicine [4] and by D. Robertson, University of Kansas [13]), (ii) cholera enterotoxin (obtained from Schwarz/Mann, Orangeburg, N.Y.), (iii) staphylococcal enterotoxin (kindly supplied by W. Beisel, Ft. Detrick, Frederick, Md. [25]), and (iv) porcine *E. coli* ST (supplied by D. Robertson, University of Kansas [1]).

Since we are interested in ultimately using this assay system to measure ST in various biological specimens, we also examined the cross-reactivities of various peptides that are found in gastrointestinal tissues and secretions. The following pure peptides were examined: synthetic human gastrin, arginine-8 vasopressin, somatostatin, cholecystokinin octapeptide, and bradykinin (all obtained from Calbiochem-Behring Corp., La Jolla, Calif.); and vasoactive intestinal peptide (obtained from Boehringer Mannheim, Indianapolis, Ind.)

Modification of the ST molecule. Since human ST contains one or more disulfide bridges which are required for biological activity (26), we assessed the influence of intact disulfide bridges on the ability of ST to bind to anti-ST. Pure ST was subjected to performic acid oxidation as perviously described (26). This procedure cleaves disulfide bridges and prevents them from reforming.

Assay of broth culture supernatants. Strains of *E. coli* known to produce ST_a, ST_b, or heat-labile enterotoxin and non-enterotoxigenic control strains of human, bovine, and porcine origins were kindly provided by the following workers: S. Formal, Walter Reed Army Institute of Research, Washington, D.C.; C. Gyles, University of Guelph, Guelph, Ontario, Canada; H. Moon, National Animal Disease Center, Ames, Iowa; R. B. Sack, Baltimore City Hospital, Baltimore, Md.; R. Guerrant, University of Virginia, Charlottesville, Va.; and D. Robertson, University of Kansas, Lawrence, Kans. All strains were grown in roller tubes in Casamino Acids yeast extract medium as previously described (7). The cultures were centrifuged, and each supernatant fluid was divided into two portions, coded, and frozen at −20°C for assay. All samples were assayed within 2 weeks of preparation. One portion was tested by the suckling mouse assay (7), and the other was tested by the RIA. Samples were coded such that the technicians performing the assays were unaware of the identity, source, or nature of the samples.

RESULTS

Radioiodination of ST and purification of ^{125}I-labeled ST. Figure 1 shows results of purification of the iodination reaction mixture by ion-exchange chromatography on diethylaminoethyl-Sephacel. Three major peaks of radioactivity were identified. The first peak, which eluted at fraction 60, represented unreacted $Na^{125}I$. Iodinated ST eluted in two peaks at fractions 103 and 112. These two peaks probably represented mono- and di-iodinated ST, respectively, and behaved identically when they were titrated against anti-ST antisera. These peaks were identified as ST by (i) their suckling mouse activities, (ii) their reactions with anti-ST antisera (see below), and (iii) comigration with unlabeled ST on thin-layer chromatography, as previously described (26). The specific activities of the ^{125}I-labeled ST preparations varied between 330 and 690 Ci/mmol, depending on the batch. The iodinated ST peaks were diluted in 120 mM sodium acetate buffer (pH 6.2) and stored at 4°C; these preparations retained full immunoreactivity for 1 month. In an attempt to prolong shelf life, samples were frozen at −20°C, but these samples showed losses of immunoreactivity as early as 2 weeks after freezing. Because of these observations, ^{125}I-labeled ST was stored at 4°C, and a fresh batch was prepared each month.

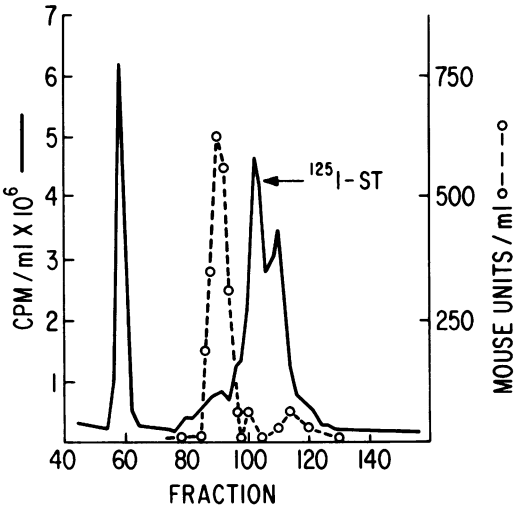

FIG. 1. *Purification of a radioiodination reaction mixture by diethylaminoethyl-Sephacel anion-exchange chromatography. A column (0.9 by 25 cm) of diethylaminoethyl-Sephacel was equilibrated with 10 mM bis-tris(hydroxymethyl)aminomethane buffer (pH 6.5) and was eluted with 600 ml of a linear NaCl gradient (0 to 200 mM) in bis-tris(hydroxymethyl)aminomethane buffer. The volume of each fraction was 2.5 ml. ^{125}I-ST, ^{125}-labeled ST.*

As shown in Fig. 1, when the various fractions were concentrated and tested by the suckling mouse assay, almost all of the ST eluted before ^{125}I-labeled ST. Thus, this column separation procedure "enriched" ^{125}I-labeled ST by separating iodinated ST from non-iodinated ST. Presumably, this occurred because the addition of iodide to the small ST molecule increased its net negative charge and slightly altered its mobility on anion-exchange chromatography columns (12).

Characteristics of goat anti-ST antisera. ST antibody was demonstrable in the two goats immunized as early as the first booster injection. Six antisera from each goat were tested for the ability to bind ^{125}I-labeled ST. Five of the six sera from each goat possessed anti-ST activity, and the antibody titers (the dilutions of serum giving 50% isotope binding) ranged from 1:8,000 to 1:40,000, depending on the date of bleeding and the batch of ^{125}I-labeled ST used. Maximum anti-ST titers were obtained after the second booster dose. Figure 2 shows the titration of one such antiserum (G108-2). Virtually all of the ^{125}I-labeled ST could be bound by this antibody, indicating that the iodination procedure did not "damage" the ST significantly. As Fig. 2 shows, as little as 0.025 μl of goat serum bound 50% of the radioligand. This represented a dilution of 1:40,000.

When varying quantities of this antibody were incubated with nonradioactive ST for 1 h at 37°C, biological activity, as assessed by the suckling mouse assay, was completely lost; 1 ml of this antiserum neutralized between 270 and 540 ng of ST (100 to 200 mouse units). Incubation of ST with nonimmune goat serum resulted in no loss of biological activity.

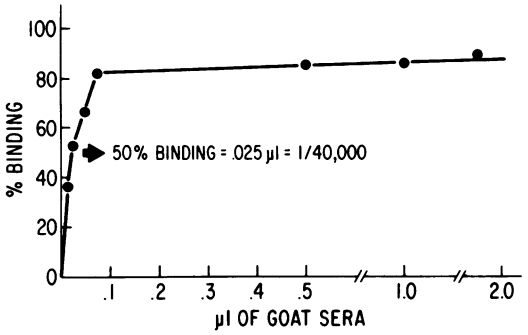

FIG. 2. *Antibody titer curve of antiserum G108-2. ^{125}I-labeled ST was incubated with increasing quantities of goat serum, as described in the text. Percent binding refers to the ^{125}I-labeled ST bound to antibody, as calculated from the percentage of total radioactivity found in the charcoal pellet and supernatant. Binding of 50% of the ^{125}I-labeled ST occurred with 0.025 µl of goat serum (an antibody titer of 1: 40,000).*

This antiserum, antiserum G108-2, was used in all of the studies described below. The dialyzed and lyophilized samples of anti-ST stored at −20°C showed no significant decreases in titer after 1 year (21).

Characteristics of the immunoassay. Figure 3 shows a typical ST 18-h radiodisplacement standard curve. As Fig. 3 shows, 50 pg of ST per assay tube could be detected with a log-linear displacement of ^{125}I-labeled ST over a range of 50 to 500 pg of ST per tube. When the reaction mixture was incubated at 4°C, equilibrium was reached by 14 to 16 h, and the preparation was stable for at least 36 h.

Other conditions examined included different buffer systems, optimal ionic strength of the assay reaction mixture, pH, and the effect of protein concentration. Of the several buffers examined, sodium acetate was superior to sodium or potassium phosphate and bis-tris(hydroxymethyl)aminomethane buffer. Maximal binding of ^{125}I-labeled ST to anti-ST antisera occurred in the ionic strength range from 100 to 200 mM. We chose a concentration of 120 mM since it provided a margin of safety at both ends of this range. Although binding was maximal in sodium acetate buffer at pH 4.0 when pure aqueous standard solutions were examined, we chose pH 6.2 for the routine assay system because this pH gave more reproducible and consistent results when biological samples were examined. Furthermore, assay performance was identical over the range from 25 to 500 µg of protein per assay tube.

Cross-reactivity studies. Figure 4 shows the abilities of various bacterial enterotoxins to cross-react in this assay system. *E. coli* heat-labile enterotoxin, staphylococcal enterotoxin B, and cholera enterotoxin did not demonstrate any cross-reactivity, even when they were tested in microgram quantities per assay tube.

In contrast, purified porcine *E. coli* ST, a 47-amino acid peptide purified from an *E. coli* strain pathogenic for pigs (1), demonstrated complete cross-reactivity in this assay system.

When the gastrointestinal peptides gastrin, cholecystokinin, bradykinin, vasopressin, somatostatin, and vasoactive intestinal peptide were tested, none demonstrated any cross-reactivity.

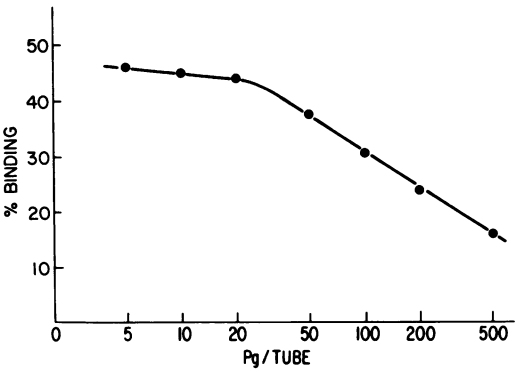

FIG. 3. *Standard curve for ST RIA, using antiserum G108-2. The total volume of the assay was 500 µl and the reaction mixture contained antibody at a final dilution of 1:40,000, ^{125}I-labeled ST (15,000 cpm), and 100 µg of bovine serum albumin in 0.12 M sodium acetate buffer (pH 6.2). Incubation was at 4°C for 18 h, and unbound ^{125}I-labeled ST was precipitated with dextran- coated charcoal. Percent binding was defined and determined as described in the legend to Fig. 2.*

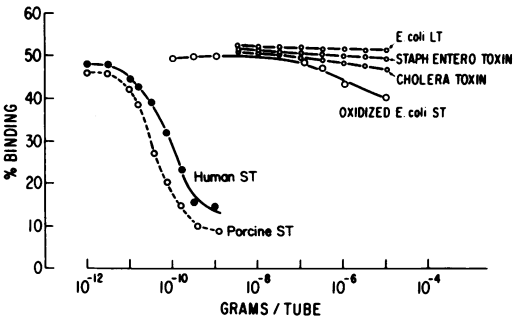

FIG. 4. *Cross-reactivity of various bacterial enterotoxins and oxidized ST with antiserum G108-2. Incubations were carried out as described in the legend to Fig. 3, and the various enterotoxins were added in the quantities shown on the abscissa. The human ST used was the ST of Staples et al. (26), and the porcine ST used was the ST of Alderete and Robertson (1). E. coli LT, E. coli heat-labile enterotoxin; STAPH ENTERO TOXIN, staphylococcal enterotoxin B.*

Effect of modifying the ST molecule. When performic acid-oxidized ST was tested (Fig. 4), it demonstrated no reactivity in the RIA system. This was true even when microgram quantities were tested.

RIA for ST in broth culture supernatant samples and comparison with the suckling mouse assay. Casamino Acids yeast extract broth supernatants of 52 ST-positive and ST-negative strains of *E. coli* were examined by both assay systems. Figure 5 shows the correlation between the two assay systems. A total of 20 strains produced only ST_a, 3 produced heat-labile enterotoxin plus ST, 3 produced only ST_b, 10 produced only heat-labile enterotoxin, and 16 were non-enterotoxigenic. As Fig. 5 shows, there was an excellent correlation between the two assay systems (correlation coefficient, 0.848). All samples that were positive in the suckling mouse assay were positive in the RIA and vice versa. This was true regardless of the origin of the strain (i.e. human, porcine, or bovine). Three *E. coli* strains which produced ST_b were examined, and all were negative.

Accuracy and reproducibility of the RIA. When an aqueous solution of pure ST was tested repeatedly on the same day, the coefficient of variation was 3.6%, and when an ST solution was measured on many separate occasions, this value was 5.7%.

When a naturally produced ST in bacterial culture medium was examined in this way, the coefficient of variation for several replicate samples ranged from 3.0 to 7.7%; for samples assayed on many separate occasions, the coefficient of variation ranged from 6.9 to 10.4%, depending on the sample.

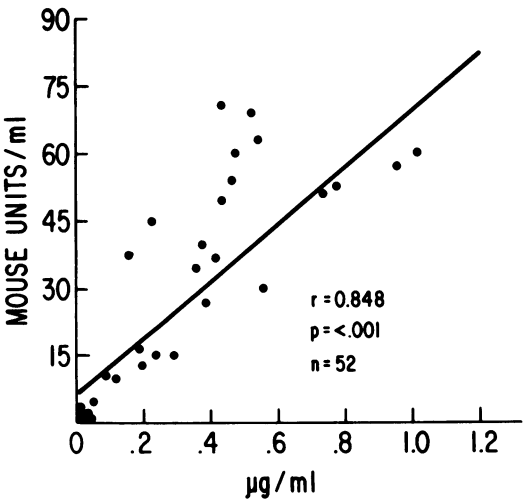

FIG. 5. *Correlation between biological and RIA measurements of ST in Casamino Acids yeast extract culture media. Biological activity was measured by the suckling mouse assay and was expressed as mouse units per milliliter, as described in the text. r, Correlation coefficient. A total of 52 E. coli strains were examined. The data for all of the negative strains are not shown.*

DISCUSSION

For a long time *E. coli* ST was thought to be poorly immunogenic. This view was based on the use of crude preparations of ST and attempts to raise antibodies by immunizing animals with peptide alone (3, 5, 9, 23). Our success in producing high-titer antisera to ST was probably due to using ST conjugated in adequate quantities to an appropriate protein carrier (i.e., bovine IgG). This hapten technique has been successful in producing antibodies to a number of other small peptide molecules (21). The production of these antisera permitted us to develop an RIA for *E. coli* ST_a which is highly sensitive and seemingly specific. This assay can detect as little as 50 pg of ST per assay tube, whereas the suckling mouse assay requires a minimum of 0.4 to 2.6 ng of ST_a (1, 26) per tube, depending on the ST_a. This is an 8- to 54-fold difference in sensitivity. In addition to this sensitivity, our RIA is seemingly specific for ST, since *E. coli* heat-labile enterotoxin, cholera toxin, and staphylococcal enterotoxin B are all unreactive in this assay system. However, this conclusion is only tentative at present since a variety of other enterotoxins remain to be tested. It will be especially interesting to examine *Yersinia enterocolitica* "ST" since this material shares some physiological and biochemical similarities with *E. coli* ST (22).

We believe that our RIA may be specific for ST_a, as defined by Burgess et al. (3). This conclusion is based on the fact that three porcine *E. coli* strains, which were provided by C. L. Gyles and H. W. Moon and were shown to produce only ST_b (i.e., active in the ligated porcine ileal loop test but inactive in the suckling mouse assay [3]), were negative when they were tested in our RIA system. This supports the hypothesis that ST_b is a different molecule than ST_a and bears little immunochemical similarity to ST_a.

In addition to measuring human ST_a, our immunoassay system measures porcine ST_a with equal sensitivity. This suggests that these two molecules, which are 18- and 47-amino acid peptides, respectively (1, 26), probably share considerable amino acid homology. A comparison of the amino acid compositions of these two ST_a molecules (1, 26) reveals that it is possible that the 18-amino acid peptide might be contained within the larger porcine ST_a, a suggestion which we have made previously (26). Some inferences

as to the immunological "active site" of the ST_a molecule can be drawn from the fact that performic acid oxidation of human ST_a completely abolishes immunolgical activity, just as it eliminates biological activity (26). This suggests that one or more disulfide bridges are involved in the immunodeterminant portion of the molecule.

Since we are interested in using this assay system to measure ST in gastrointestinal tissues and secretions, it was important to assess whether various peptides found in these biological tissues cross-react with our anti-ST antiserum. None of the six gastrointestinal peptides examined thus far, which are approximately the same size as ST_a and bear some structural similarity to ST_a, demonstrated any cross-reactivity.

Many RIAs for peptide hormones measure immunological activity rather than biological activity, an important distinction when trying to draw conclusions about the biological relevance of finding the material in a particular specimen. We are not certain that our assay measures biological ST activity. The finding of an excellent correlation between biological activity (suckling mouse activity) and immunological activity is consistent with this possibility. Alternatively, it is also possible that this assay measures immunological ST activity which is secreted in direct proportion to a molecule with full biological activity. Further work will be required to settle this issue.

At present, the most commonly used assay for the detection of ST_a is the suckling mouse assay (7). Unfortunately, this is a bioassay and has all of the faults of such techniques; i.e., it is cumbersome, expensive, inconvenient, and only semiquantitative. We believe that the development of our RIA will overcome these difficulties. Our RIA is substantially more sensitive than the suckling mouse assay and is specific, reproducible, and easy to perform. However, it does require that a fresh, fully immunogenic batch of ^{125}I-labeled ST be prepared each month. In addition to these attributes, this RIA can be used to detect and quantitate ST_a in complex bacterial culture media, as shown in Fig. 5. ST_a does not have to be extracted from the culture media before assay since the sensitivity of the assay permits substantial dilution of the culture media, so that potential interfering substances are also diluted. In assays of Casamino Acids yeast extract broths containing ST_a, we begin by assaying a 1:500 dilution of the broth.

We now plan to use this RIA for ST_a to detect and measure ST_a directly in stools. If successful, this would greatly simplify the detection of cases and might even serve as a rapid diagnostic test. When the suckling mouse assay was used to detect ST_a directly in stools, ST was found in only 36% of the cases from which ST-producing *E. coli* strains were isolated (17). With a more sensitive assay, the percentage of ST detection will probably increase substantially. In addition to the possibility of using this RIA for ST_a as a rapid and sensitive diagnostic test, this procedure should (i) make feasible large-scale epidemiological studies of the role of ST-producing bacteria in acute diarrheal disease, (ii) permit the performance of physiological studies concerning the anatomicocellular site of ST action, etc., and (iii) provide a tool to assess the immunochemical differences and similarities among the various STs that have been reported (1, 3, 10, 18).

ACKNOWLEDGMENTS

This work was supported by Veterans Administration Research Project 596-3108-01 and by Research Contract 223-77-2115 from the Food and Drug Administration.

We acknowledge the expert assistance of Danny Walls and the secretarial assistance of Margaret Doerflein.

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